

Cytomegalovirus Capsid Protease: Biological Substrates Are Cleaved More Efficiently by Full-Length Enzyme (pUL80a) than by the Catalytic Domain (Assemblin)[▽]

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We compared the full-length capsid maturational protease (pPR, pUL80a) of human cytomegalovirus with its proteolytic domain (assemblin) for the ability to cleave two biological substrates, and we found that pPR is more efficient with both. Affinity-purified, refolded enzymes and substrates were combined under defined reaction conditions, and cleavage was monitored and quantified following staining of the resulting electrophoretically separated fragments. The enzymes were stabilized against self-cleavage by a single point mutation in each cleavage site (ICRMT-pPR and IC-assemblin). The substrates were pPR itself, inactivated by replacing its catalytic nucleophile (S132A-pPR), and the sequence-related assembly protein precursor (pAP, pUL80.5). Our results showed that (i) ICRMT-pPR is 5- to 10-fold more efficient than assemblin for all cleavages measured (i.e., the M site of pAP and the M, R, and I sites of S132A-pPR). (ii) Cleavage of substrate S132A-pPR proceeded M>R>I for both enzymes. (iii) Na₂SO₄ reduced M- and R-site cleavage efficiency by ICRMT-pPR, in contrast to its enhancing effect for both enzymes on I site and small peptide cleavage. (iv) Disrupting oligomerization of either the pPR enzyme or substrate by mutating Leu382 in the amino-conserved domain reduced cleavage efficiency two- to fourfold. (v) Finally, ICRMT-pPR mutants that include the amino-conserved domain, but terminate with Pro481 or Tyr469, retain the enzymatic characteristics that distinguish pPR from assemblin. These findings show that the scaffolding portion of pPR increases its enzymatic activity on biologically relevant protein substrates and provide an additional link between the structure of this essential viral enzyme and its biological mechanism.

All herpesviruses encode a maturational protease that acts during nucleocapsid formation and is required to produce infectious progeny virus. The human cytomegalovirus (HCMV) homolog is the product of open reading frame (ORF) UL80a and is synthesized as a 74-kDa protein (pPR, pUL80a). It is organized as an amino-28-kDa proteolytic domain called assemblin, joined by an 8-kDa “linker” to a carboxy-46-kDa scaffolding domain (Fig. 1). All homologs share this general organization.

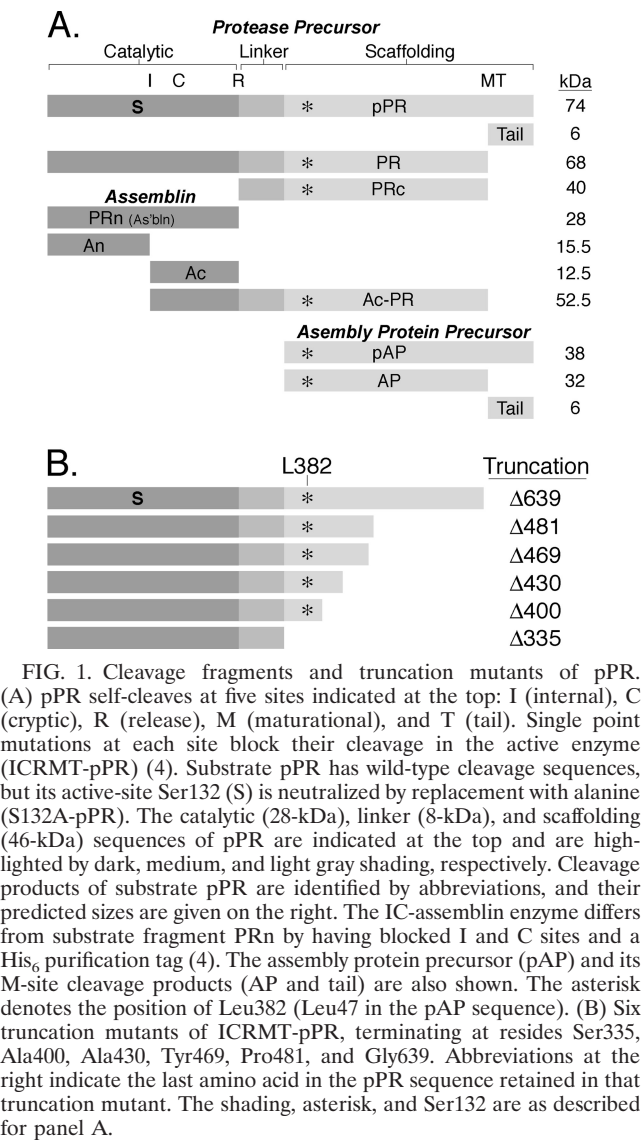
The 2.1-kb ORF that encodes the HCMV protease also encodes a protein about half its size, called the assembly protein precursor (pAP; 38 kDa), which is independently and more abundantly expressed from the in-frame, 3′-coterminal UL80.5 ORF (18, 37). Together, these proteins coordinate the earliest organizational and maturational stages of capsid formation (5, 14), including self-interactions to form homo-oligomers (19, 41), interactions with each other to form hetero-oligomers (26, 41), and interactions with the major capsid protein (MCP; pUL86) to form larger complexes destined to become subunits of the capsid (3, 12, 25, 41). Many of these interactions occur in the cytoplasm, including those with MCP that enable its translocation into the nucleus via nuclear localization sequences within the UL80 proteins (24, 26).

Once inside the nucleus, pPR and pAP are proposed to further interact, causing MCP protomers to coalesce with themselves and with other capsid subunits (e.g., triplex and portal) to form the procapsid shell (5, 14, 31). Although the protease is not absolutely required to form a capsid-like structure (32, 34), its function is essential for eliminating the internal scaffolding proteins (i.e., pAP and pPR) to make room for the viral DNA (13, 27, 36). Mechanisms to deliver the protease to the interior of the procapsid and prevent its premature activation are needed to ensure efficiency in the process. Targeting of pPR to the capsid interior is attributed to the pAP sequence included in its scaffolding portion (Fig. 1) (3, 17, 33, 41), but little is known about how its activity is sequestered until needed.

Once activated, all herpesvirus protease homologs cleave themselves at two highly conserved sites, called the maturational (M) site and the release (R) site (Fig. 1) (39). M-site cleavage is faster, both in cells transfected with the cloned protease gene (16, 38) and in virus-infected cells (16). The M site is present near the carboxyl end of both pPR and pAP, and its cleavage breaks the interaction of the internal scaffolding proteins with MCP at the interior face of the capsid shell. R-site cleavage releases assemblin from the rest of the protein and is required to produce infectious virus (22). It remains to be determined whether it is needed for enzymatic reasons (e.g., altering or mobilizing activity) or structural reasons (e.g., reducing the size to eliminate from shell) or both. In addition to these two herpesvirus group-conserved cleavage sites, the HCMV pPR contains three others: two

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within assemblin, called the internal (I) site and cryptic (C) site, and one in the 6-kDa tail domain (T site). All three are shown in Fig. 1 and have been discussed elsewhere in greater detail (4, 8, 15, 16, 19).

Information about enzymatic and structural characteristics of the herpesvirus protease has come mainly from studies using assemblin. Site-directed mutagenesis and crystallography established that it is a serine protease but, at variance with the typical Ser-His-Asp catalytic triad, uses a histidine in place of the third-position aspartic acid (9, 28, 30, 35, 38). The eight-strand beta-barrel organization of assemblin is also atypical, although it orients the catalytic triad residues in a spatial configuration like that of other serine proteases (9, 28, 30, 35). Assemblin monomers dimerize to gain enzymatic activity (10, 11, 20), and the interaction results in conformational changes that reposition a critical arginine to stabilize the transition state in the active site (1, 6). The active dimer has two independently functioning catalytic sites, one on each subunit, separated by ~35 Å (9, 21, 28, 30, 35).

With relatively little information available for the full-length protease (pPR), assemblin has been the only model available for considering mechanisms that regulate its activation, designing active site cleft-based inhibitors, and screening for inhibitors in high-throughput assays. However, given that the earliest interactions and trafficking of the protease occur before its cleavage to release assemblin and that pPR and assemblin may differ in functionally significant ways, the importance of learning more about pPR has become increasingly evident, and we and others developed methods to express, purify, and begin studying it *in vitro* under defined conditions (4, 40).

Our initial studies of pPR *in vitro* used a gradient sedimentation refolding step coupled with a fluorogenic small-peptide cleavage assay and provided information about both oligomerization and activity. Structurally, pPR showed a major difference from assemblin by forming putative tetramers (versus dimers for assemblin) through a dominant self-interaction sequence called the “amino-conserved domain” within its scaffolding portion (Fig. 1) (4, 19). Enzymatically, however, pPR and assemblin showed comparable activities in initial assays using a small peptide substrate (4). Although an earlier study using purified proteins concluded that pPR is substantially less active than assemblin (40), cell-based comparisons provided no evidence of that (16), and the difference has been attributed to procedural details (4).

The work reported here was done to follow up on our initial peptide-based enzymatic comparisons of pPR with assemblin, using more biologically representative substrates that may be recognized and cleaved more specifically or differently than small peptides. We used the two primary protein substrates of the CMV UL80a protease: (i) the assembly protein precursor (pAP, pUL80.5), which contains only the M and T sites, and (ii) pPR itself, containing all five native cleavage sites but rendered proteolytically inactive (S132A-pPR). We affinity purified the two substrates and used them in time course cleavage reactions to compare the enzymatic properties of similarly purified, self-cleavage-blocked pPR (ICRMT-pPR) and assemblin (IC-assemblin). Our results demonstrate that pPR is more efficient than assemblin at cleaving both protein substrates.

MATERIALS AND METHODS

Plasmids. Details of constructing the pET-17b expression plasmids encoding IC-assemblin (EB84) and ICRMT-pPR (EB87) have been described before (4). The I and C cleavage sites of assemblin and the I, C, R, M, and T cleavage sites of pPR were altered by point mutations that stabilized them against autoproteolysis (IC-assemblin and ICRMT-pPR). The enzymatically inactive pPR used here as substrate (S132A-pPR; EB24) and the self-interaction-impaired pPR (L382A-pPR; EB88) have also been described previously (4, 19). Leu382 in the amino-conserved domain of substrate pPR was replaced with alanine, by using site-directed mutagenesis (catalog number 200518, QuikChange; Agilent Technologies, La Jolla, CA) to change codon CTA to GCT, yielding the mutant S132A/L382A-pPR. The HCMV pAP (pUL80.5) gene, including the coding sequence for a carboxyl six-histidine (His₆) tag, was PCR amplified from plasmid KC3 and ligated into pET-17b using restriction sites HindIII and NdeI (pAP-6His). The complete coding sequences of the resulting S132A/L382A-pPR and Wt-pAP were verified.

Protein expression and recovery. These procedures were done as described more completely before (4). Proteins were expressed in *Escherichia coli* strain BL21(DE3)pLysS (catalog number 70232; Novagen/EMJ, Gibbstown, NJ). At an optical density at 600 nm of ~0.6, cultures were induced by adding isopropyl β-D-1-thiogalactopyranoside (1 mM; catalog number 15529-019; Invitrogen, Carlsbad, CA), and incubation at 37°C was continued for 4 h. Bacteria were

collected by centrifugation and frozen at -80°C until ruptured by using a French pressure cell. Protein inclusions were recovered from the resulting lysate and dissolved in 8 M urea, and the resulting solutions were cleared of larger material by centrifugation at $100,000 \times g$ (28,500 rpm in a Beckman SW41Ti rotor) for 30 min at 4°C .

The His₆-tagged proteins were recovered from the clarified solutions by immobilized metal affinity chromatography (IMAC) using Ni²⁺-charged Sepharose beads (catalog number 17-5318-01; GE Healthcare, Piscataway, NJ) for binding and 300 mM imidazole in 2 M urea for elution. Proteins in the IMAC elution fractions were identified and quantified by separation and staining in polyacrylamide gels (4), and fractions containing the highest protein concentrations were stored at -80°C . Proteins were refolded from the 2 M urea IMAC elution fractions by two-step dialysis or renaturation centrifugation (4), as indicated.

Cleavage assays. Refolded enzymes and substrates were combined in a molar ratio of 1:5 (enzyme:substrate) and the total amount of protein was ~ 3 to 5 μg . Incubation volumes were 144 μl , reactions were done at room temperature ($\sim 20^{\circ}\text{C}$), and the times of reaction are specified. Reactions were done in either cleavage buffer (CB; 10% glycerol, 10 mM dithiothreitol [DTT], 500 mM Na₂SO₄, 100 mM morpholinepropanesulfonic acid buffer, pH 7.2) or gradient buffer (GB; 300 mM NaCl, 15% glycerol, 50 mM DTT, 50 mM phosphate buffer [pH 8.0]), both of which were used in an earlier study (4).

Samples withdrawn from the master reaction tube were combined 3:1 with protein sample buffer (3 parts NuPAGE 4 \times LDS [catalog number NP0007; Invitrogen, Madison, WI] plus 1 part 1.0 M DTT) and stored at -80°C until analyzed. Some protein binding to the reaction tubes occurred with incubations longer than ~ 2 h.

Polyacrylamide gel electrophoresis, protein staining, and quantification. Proteins and fragments in the cleavage reaction mixtures were separated by electrophoresis in 4-to-12% polyacrylamide gradient gels (catalog number NP0323BOX; Invitrogen) containing sodium dodecyl sulfate (SDS) and buffered with 2-CN-morpholinoethanesulfonic acid (MES; catalog number NP0002; Invitrogen) (SDS-PAGE). Protein molecular weight markers were SeeBlue Plus2 (catalog number LC5925; Invitrogen). After electrophoresis, proteins in the gels were detected by staining with Coomassie brilliant blue (CBB) and imaged and quantified using a Kodak Gel Logic 200 system (ClearStream, Rochester, NY). Protein amounts were calculated by interpolating from known concentrations of bovine serum albumin as a standard (4).

RESULTS

The herpesvirus maturational protease (pPR) cleaves itself and the more abundant assembly protein precursor (pAP) as natural substrates. We used both to compare the enzymatic activities of purified HCMV pPR and assemblin in assays based on the production of specific cleavage fragments resolved by SDS-PAGE and quantified following protein staining. The enzymes were stabilized against self-cleavage by point mutations in the I, C, R, M, and T cleavage sequences to yield ICRMT-pPR and IC-assemblin, and the substrate form of pPR was rendered proteolytically inactive by replacing its active-site serine with alanine (S132A-pPR). The enzyme and substrate proteins used in this work, and the fragments resulting from their cleavage, are summarized in Fig. 1 for reference.

pAP was cleaved faster by pPR than by assemblin. pAP was combined with purified ICRMT-pPR or IC-assemblin and incubated for 3 h. Samples taken from the reaction mixtures were subjected to SDS-PAGE and protein staining. Cleavage of pAP to AP was faster and more complete for pPR than assemblin (Fig. 2, No Na₂SO₄). Calculations based on measurements from these images showed that pPR cleaved 50% of the substrate in ~ 15 min, versus ~ 100 min for assemblin (~ 7 -fold faster) (Fig. 3, arrows). The 6-kDa tail fragment cleaved from pAP increased in correspondence with AP (Fig. 2).

As part of the same reaction set, we tested the effect of adding 0.5 M Na₂SO₄, a cosmotrope that enhances assemblin

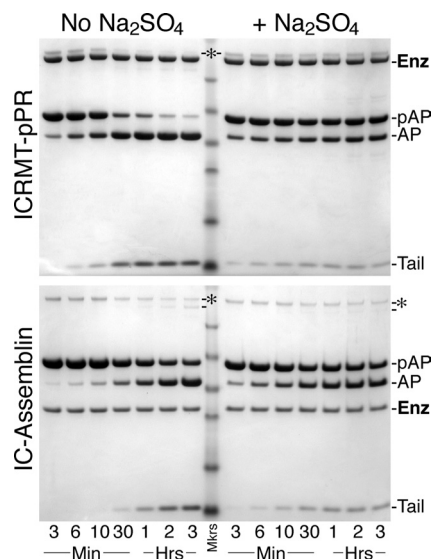


FIG. 2. M-site cleavage of pAP by ICRMT-pPR and IC-assemblin. Enzymes ICRMT-pPR or IC-assemblin were combined with substrate (pAP) in a 1:5 molar ratio and incubated without (left panels) or with (right panels) Na₂SO₄. Samples taken at the indicated times were subjected to SDS-PAGE, and proteins were stained with CBB. Samples and gels were prepared and analyzed in parallel. **Enz** identifies the enzyme band; other abbreviations to the right of the image are as described for Fig. 1A. The band indicated by an asterisk is a dimer of pAP, and the band immediately beneath it (see dash, lower image) is a dimer of AP that comigrated with pPR in the top panel.

activity in peptide cleavage assays (4, 10, 11, 20). In contrast to its enhancing effect on the cleavage of a small peptide substrate (4), Na₂SO₄ decreased the final extent of pAP cleavage for both enzymes (50% for pPR and 10% for assemblin), even though it slightly accelerated initiation of the reactions (Fig. 2, + Na₂SO₄).

S132A-pPR is cleaved faster by pPR than by assemblin. pPR contains all five of its known cleavage sequences, enabling a proteolytically inactive form of itself to serve as a single substrate with which all cleavages can be simultaneously monitored. We used mutant S132A-pPR for this purpose and evaluated its cleavage following reaction with ICRMT-pPR or IC-assemblin. Purified S132A-pPR and enzyme were combined, reacted with or without 0.5 M Na₂SO₄, and analyzed as described above for pAP. pPR was more efficient than assemblin at cleaving the M, R, and I sites of substrate S132A-pPR, with or without Na₂SO₄, and the pattern of cleavage products differed for each of the four reactions (Fig. 4A and B).

(i) M-site cleavage of S132A-pPR (74 kDa) yields two fragments, PR (68 kDa) and tail (6 kDa). PR is unsuitable as a measure of M-site cleavage because it contains an additional cleavage site (Fig. 1, R site) and accumulates only transiently before becoming a substrate itself. The tail fragment also contains an additional cleavage site (T site), but we have found no evidence that it is a substrate in these reactions and therefore used its appearance and accumulation as measures of the timing and extent of M-site cleavage.

With ICRMT-pPR as the enzyme and no Na₂SO₄, M-site cleavage reached 50% by 6 min and completion by ~ 30 min (Fig. 4A and C and 5). The amount of tail fragment increased

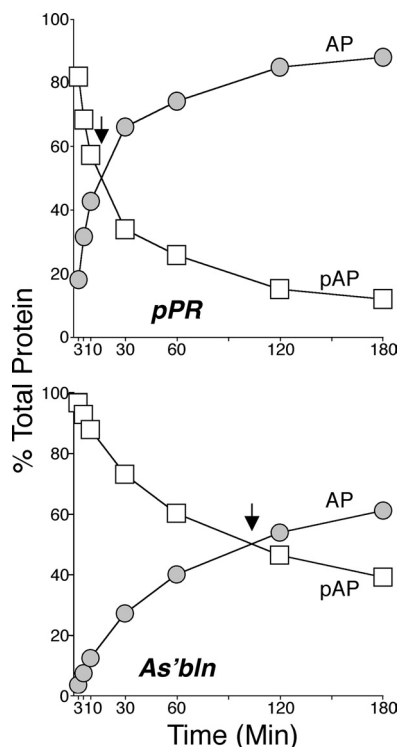


FIG. 3. pAP cleavage is faster with pPR than assemblin. Data from Fig. 2 (No Na_2SO_4) were used to graph the percentage of substrate (pAP) versus product (AP) in samples taken at the indicated times from reactions with ICRMT-pPR (pPR) and IC-assemblin (As'bln). Arrows indicate points at which amounts of precursor (pAP) and product (AP) are equal.

with that of the PR up to 30 min but then decreased as R-site cleavage converted it to PRn+PRc (Fig. 1, 4A, and 5). The ICRMT-pPR enzyme is stable to autolysis and persisted at the position of pPR throughout the reaction (Fig. 4A, **Enz**). Under the same reaction conditions (No Na_2SO_4), IC-assemblin was at least 6-fold slower than ICRMT-pPR, showing 50% cleavage at ~60 min (Fig. 4A to C). It took 30 min for assemblin to yield an amount of Tail fragment comparable to that reached by ICRMT-pPR in 3 min, and still longer (1 to 5 h) to achieve exhaustive cleavage of S132A-pPR M sites (i.e., the most Tail and least pPR) (Fig. 4B and C).

(ii) R-site cleavage of PR produces a stable carboxyl fragment (PRc; 40 kDa) and an unstable amino fragment (PRn, assemblin; 28 kDa) that is subsequently cleaved to give fragments An and Ac (Fig. 1). In the reaction with ICRMT-pPR (No Na_2SO_4), PRc and PRn were detected by 3 min and continued to accumulate throughout the reaction, unlike the Tail fragment, which reached a maximum amount in 30 min (Fig. 4 and 5). R-site cleavage was ~10-fold slower for assemblin, based on the accumulation of PRc. Assemblin required approximately 5 and 15 h, respectively, to release amounts of PRc comparable to those produced by pPR in 30 min and 1 h (Fig. 4D). The rate of PRc accumulation in the assemblin reaction was nearly constant between 1 and 15 h, in contrast to the decreasing rate observed with pPR over that interval, as the PR substrate is consumed and the reaction nears completion. The PRn cleavage product (no His₆ tag) and the IC-

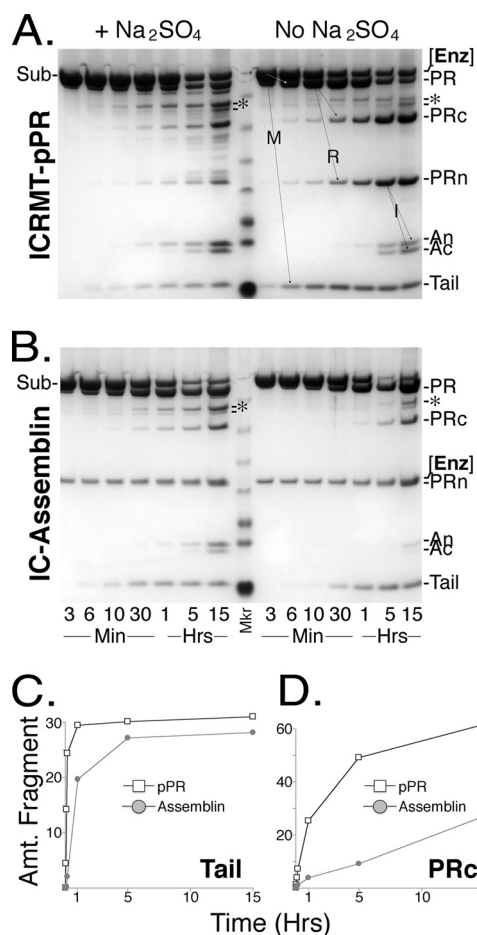


FIG. 4. Cleavage of substrate-pPR by enzymes pPR and assemblin. Substrate pPR (Sub; S132A-pPR) was reacted with protease (**Enz**; ICRMT-pPR or IC-assemblin), with or without Na_2SO_4 added, as indicated at the top. (A and B) Reaction products in samples taken at the times shown, after separation by SDS-PAGE and staining with CBB. Samples and gels were prepared and analyzed in parallel. Abbreviations at right are as described for Fig. 1A; asterisks indicate the position of the putative Ac-PR fragment. The letters M, R, and I and arrows indicate products resulting from the respective cleavages. The center lanes contain a mixture of marker proteins (Mkr). (C and D) Data in the No Na_2SO_4 lanes of panels A and B, respectively, were quantified to compare the kinetics of M- and R-site cleavage for pPR and assemblin, based on relative amounts of tail or PRc (plotted in arbitrary units).

assemblin enzyme (with His₆ tag) are too close in size to be resolved in these SDS-PAGE separations, but the presence of PRn is evident from the increased intensity of the PRn/[Enz] band in the 15-h sample (Fig. 4B).

(iii) I site. As the amount of PRn increased late in the reaction with pPR, it became a substrate for I-site cleavage (Fig. 4A and 5). Approximately equimolar amounts of its cleavage products, An (15.5 kDa) and Ac (12.5 kDa), were present by 5 h and more abundant by 15 h (Fig. 4A and 5). The small amount of An detected before Ac in these reactions (Fig. 4A, No Na_2SO_4) is thought to originate from I site cleavage of PR or possibly pPR, yielding fragments An+Ac-PR (52.5 kDa) (Fig. 1) and/or Ac-PR-Tail (58.5 kDa). Consistent with this

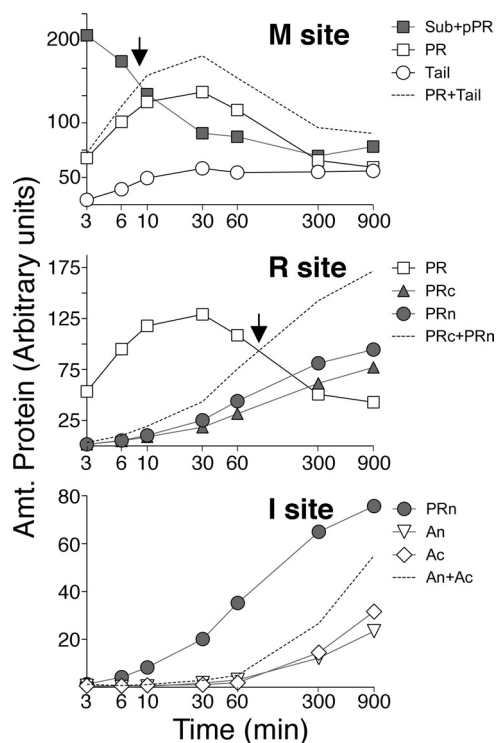


FIG. 5. Kinetics of S132A-pPR M-, R-, and I-site cleavages by ICRMT-pPR. Data from Fig. 4A (no Na_2SO_4) were quantified and plotted to show the relative kinetics of M-, R-, and I-site cleavage by pPR. The precursor and resulting two fragments are plotted for each cleavage reaction. Substrate and enzyme forms of pPR comigrated during SDS-PAGE (see Fig. 4A and B, Sub and Enz) and were measured together (Sub + pPR) in the top panel. The time is shown on a \log_{10} scale to resolve the early data points. Fragment abbreviations are as for Fig. 1A. Dashed lines show summed data points for the two product fragments in each panel. Arrows indicate the point at which the amount of precursor equals the amount of products.

interpretation, a fragment in the size range of Ac-PR was detected by 6 min of reaction (Fig. 4A and B and 6, asterisks).

Like M- and R-site cleavage, I site cleavage was slower with assemblin than with pPR. Fragments An and Ac were detected with confidence only after 15 h in the reaction with assemblin (Fig. 4B, No Na_2SO_4), whereas comparable amounts were present in the pPR reaction by 1 h (Fig. 4A, No Na_2SO_4). The only other protein consistently observed in these reactions in the absence of Na_2SO_4 ran just ahead of the putative Ac-PR fragment. Its size is compatible with it being the carboxyl end of PR resulting from C-site cleavage of either PR or Ac-PR (i.e., 3 kDa smaller than Ac-PR) (Fig. 4A and B, dash below asterisks). It was present in the 3-min sample and remained constant in amount until increasing in the 15-h sample.

(iv) Na_2SO_4 . Adding 0.5 M Na_2SO_4 to the reaction mixtures, as done to increase assemblin activity in peptide cleavage assays (4, 10, 11, 20), changed the protein cleavage pattern of both enzymes in different ways. Most notable was the reduction it caused in R-site cleavage by pPR, as reflected by the slower accumulation of fragments PRc and PRn (Fig. 4A). M-site cleavage by pPR was also slowed, as it was with the pAP substrate (Fig. 2 and 4A). Second, Na_2SO_4 had a minimal effect on the initiation of M- and R-site cleavage by pPR but

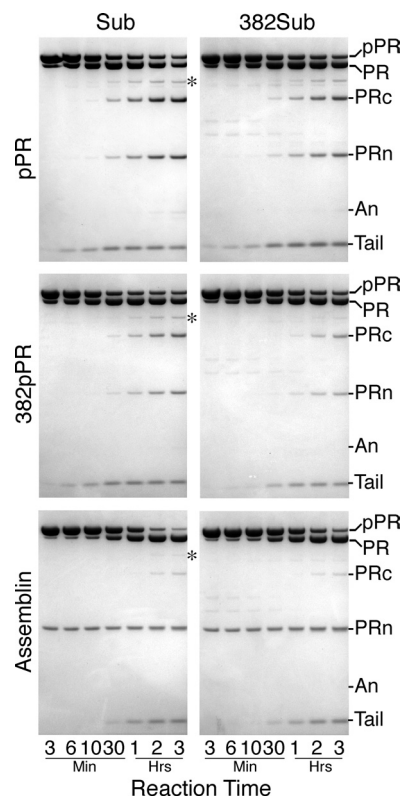


FIG. 6. L382A mutation in either pPR or substrate reduces cleavage efficiency. Wild-type and L382A mutant protease (pPR and 382pPR) were reacted with wild-type and L382A mutant substrate (Sub and 382Sub) in four pairs: pPR/Sub, pPR/382Sub, 382pPR/Sub, and 382pPR/382Sub. Reactions of assemblin with wild-type substrate and with mutant substrate were included for comparison (Assemblin/Sub and Assemblin/382Sub). Shown here are the reaction products, separated by SDS-PAGE and stained with CBB. Samples and gels were prepared and analyzed in parallel. Reaction times are indicated at the bottom; the cleavage fragments identified in Fig. 1A are labeled on the right. The asterisk indicates putative fragment Ac-PR. Faint bands between PRc and PRn in reactions with the L382A/S132A-pPR substrate (382Sub) were peculiar to this substrate preparation and without effect on the reaction (data not shown from similar reactions with mutant substrate preparations lacking these bands).

accelerated both for assemblin (Fig. 2 and 4). Third, Na_2SO_4 enhanced I-site cleavage for both pPR and assemblin, as evidenced by the stronger intensities of the An and Ac bands in the 15-h samples, and increased amounts of the putative Ac-PR band (Fig. 4A and B). Fourth, additional protein bands appeared between PR and PRc and between PRc and PRn in the pPR reaction with Na_2SO_4 (Fig. 4A), suggesting that the fidelity of cleavage may be reduced by this salt.

Thus, Na_2SO_4 (i) slowed M- and R-site cleavage by pPR, (ii) promoted earlier initiation of M- and R-site cleavage by assemblin but slowed overall accumulation of their products, and (iii) promoted earlier and more extensive I-site cleavage for both enzymes, with apparently more nonspecific cleavage.

Relative kinetics of M-, R-, and I-site cleavages by pPR. The relative kinetics of S132A-pPR cleavage by ICRMT-pPR were calculated from the data in Fig. 4A (No Na_2SO_4) and are summarized in Fig. 5. M-site cleavage was characterized by an immediate decrease in the amount of S132A-pPR (Fig. 5,

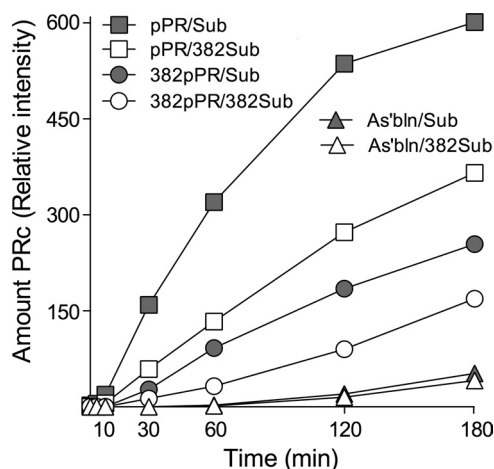


FIG. 7. Kinetics of PRc formation with the L382A mutation in the pPR enzyme or substrate. The PRc bands in each panel of Fig. 6 were quantified, and the data are summarized here. Abbreviations are as for Fig. 6. Filled symbols are results of reactions with “wild-type” substrate; empty symbols are results of reactions with L382A mutant substrate.

Sub+pPR), accompanied by the immediate appearance and accumulation of its two product fragments, PR and Tail (top panel). Within the first 8 min of reaction, 50% of the substrate had been cleaved (Fig. 5, top panel, arrow). By 30 min the amount of Tail fragment reached a maximum, indicating M site cleavage was complete, and the amount of PR began decreasing due to cleavage at its R site. R-site cleavage was identified and quantified by the appearance and amount of stable fragment PRc and was accompanied by an increase in the amount of fragment PRn (unstable intermediate). By 90 min, 50% of PR had been cleaved to PRc+PRn (Fig. 5, middle panel, arrow). I-site cleavage is identified diagnostically most often by conversion of PRn to equimolar amounts of fragments An and Ac (2, 7, 15). It initiated and proceeded much more slowly and without an appreciable increase in rate until >1 h, as the precursor fragment PRn reached higher concentrations (Fig. 5, bottom panel). Thus, the order of cleavage in these reactions was M>R>I, with no basis for ranking C or T site cleavage.

Disrupting self-interaction of pPR reduces efficiency of cleavage reaction. Point mutation L382A in the amino-conserved domain of the UL80 proteins (L47A in the UL80.5 sequence [41]) disrupts the self-interaction of pPR (4, 19) and reduces its enzymatic activity in assays using a small-peptide substrate (4). We showed here that this mutation also reduces the enzymatic efficiency of pPR in reactions with its protein substrate, S132A-pPR.

Leu382 was replaced with alanine in both the ICRMT-pPR enzyme (ICRMT/L382A-pPR) and the S132A-pPR substrate (S132A/L382A-pPR), and pair-wise combinations of wild-type and mutant enzymes and substrates were reacted. Samples taken at the indicated times during a 3-h reaction period were subjected to SDS-PAGE and CBB staining (Fig. 6), and accumulation of the stable R-site cleavage fragment PRc was quantified for all reactions (Fig. 7).

The mutation reduced reaction efficiencies, whether in the

enzyme or the substrate or both, showing a comparatively stronger effect in the enzyme than in the substrate. Relative to the wild-type protease/substrate pair (pPR/Sub), after reaction for 3 h the amount of PRc produced was 40% lower with the mutation in the substrate only (pPR/382Sub), 60% lower with the mutation in the enzyme only (382pPR/Sub), and 70% lower when in both (382pPR/382Sub) (Fig. 6). R-site cleavage by assemblin, which terminates before the amino-conserved domain, showed little change using the mutant substrate (Fig. 6 and 7, As'bln/382Sub versus As'bln/Sub).

Sequences downstream of the amino-conserved domain are less critical for enzymatic activity. Leu382 is located in the scaffolding portion of pPR (Fig. 1, asterisk), within the 19-amino-acid amino-conserved domain (41). To determine whether there are sequences further downstream that impact enzymatic activity, we made a set of six truncation mutants extending from the carboxyl end of pPR toward and through the amino-conserved domain (Fig. 1B) and tested them with substrate S132A-pPR in cleavage assays as described above. ICRMT-pPR and IC-assemblin were included for comparison.

Cleavage products of the reactions are shown in Fig. 8A to C, and quantification of the PRc, PRn, and tail fragments for each enzyme is shown in Fig. 8D. Mutants 639, 481, and 469, missing 22%, 71%, and 75% of the scaffolding sequence downstream of the amino-conserved domain, showed enzymatic characteristics of pPR, including (i) robust cleavage at both the M site (producing tail) and R site (producing PRc and PRn) and (ii) relatively more PRc and PRn than tail (Fig. 8D). Mutants 430 and 400, missing 87% and 96% of the scaffolding sequence downstream of the amino-conserved domain, behaved more like assemblin, showing (i) comparable M-site cleavage, with a yield of Tail fragment similar to that of pPR, but (ii) relatively poor R-site cleavage, with yields of PRc and PRn much lower than with pPR (Fig. 8D). Remarkably, truncation mutant 335 (Fig. 1B, assemblin + linker), missing all of the amino-terminal conserved domain, showed only trace activity in these short reactions (i.e., perceptible PR band in some gels) and notably less activity than assemblin, even with longer reaction times (Fig. 8C and data not shown for longer reaction times).

DISCUSSION

Most of our information about the herpesvirus capsid protease has come from studies done with its catalytic fragment assemblin (PRn; 28 kDa), which was readily expressed and purified and has been crystallized, characterized enzymatically, and used in high-throughput screens to identify potential inhibitors. The full-length precursor protease (pPR; 74 kDa) was comparatively intractable in such studies and received less attention. However, pPR is required for the production of infectious virus, and assemblin alone is unable to substitute for it during replication. Defining the biological mechanism of pPR by identifying and studying the essential features that distinguish it from assemblin will lead to an understanding of its role in herpesvirus capsid formation and maturation and provide additional insight into inhibiting its activity.

In an earlier report we described methods and assays used to prepare and study purified full-length pPR *in vitro* (4). That work revealed structural differences between pPR (putative

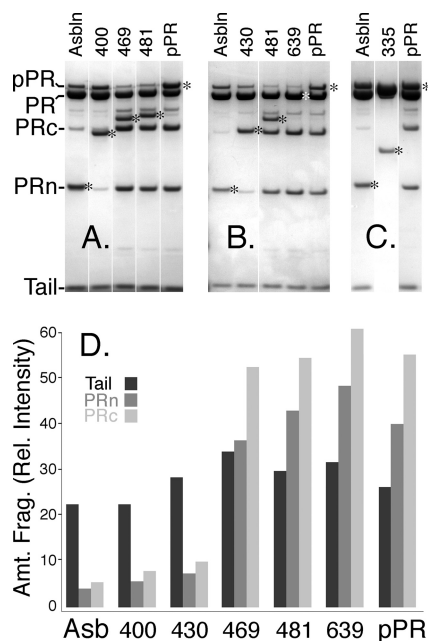


FIG. 8. Carboxyl truncations approaching Leu382 of pPR reduce proteolytic activity. (A to C) Truncation mutants of pPR terminating with residues Ser335, Ala400, Ala430, Tyr469, Pro481, and Gly639 (just upstream of the M site; VNA₆₄₃) (Fig. 1B) were reacted with S132A-pPR substrate for up to 2 h, and the resulting products were separated by SDS-PAGE and visualized by CBB staining. Data from three separate experiments, each including control reactions with pPR and assemblin (AsbIn), are presented as collages. Enzyme designations are given at the top of each lane; corresponding enzyme bands are indicated by asterisks. (D) Relative amounts of the tail, PRn, and PRc fragments for each of the cleavage reactions. Values for assemblin, pPR, and deletion mutants 400, 469, 481, and 639 were calculated from data in panel A, and values for truncation mutant 430 were calculated from data in panel B; the intensities of these bands were too weak to measure for truncation mutant 335 (panel C). Values in panel D for the PRn band in the assemblin reaction (A) and the PRc band in the reactions of mutants 400 and 430 (A and B, respectively) could not be obtained from direct measurements (the enzyme comigrated with the fragment). They were therefore calculated from values for the corresponding PRc and PRn bands by using the relationship PRc/PRn = 1.39. The 1.39 ratio of PRc to PRn was calculated from measurements made from the 469, 481, and pPR lanes.

homotetramer) and assemblin (homodimer) but provided no evidence for enzymatic differences. Since those initial comparisons used a small-peptide mimic of the M-site sequence as substrate, and considering that pPR and assemblin may behave differently with the proteins they target during virus infection, we developed an alternate *in vitro* assay using their biological substrates, pAP (e.g., pAP-His₆) and pPR (e.g., S132A-pPR-His₆).

By applying this assay, we found that pPR is much more active than assemblin at cleaving both proteins. For M-site cleavage, pPR consistently showed at least 5-fold-better activity than assemblin, with either pAP or S132A-pPR as substrate. This difference is due at least in part to interactions through the scaffolding sequence present in pPR and both substrate proteins, as discussed below. Assemblin, which lacks this sequence, is disadvantaged for interacting with pPR and pAP. Similarly, a peptide substrate lacking the scaffolding sequence

would be compromised in its interactions with pPR, resulting in a more comparable rate of cleavage for the two enzymes, as observed in our fluorogenic peptide assay (4).

The activity difference between pPR and assemblin was even greater for R-site cleavage than for M-site cleavage, with pPR being on the order of 10-fold more efficient. This distinguishing characteristic of pPR (Fig. 8) may reflect its role in initiating cleavage during capsid maturation. As the form of the protease incorporated into capsids (29), pPR must make the initial R-site cleavage to release assemblin from its scaffolding sequence. Preceding M site cleavage would dissociate PR from potentially restricting interactions with other proteins (e.g., major capsid protein, and perhaps portal protein). An enhanced capacity for pPR to accommodate and cleave both the M and R sites could account for an active-site difference between it and assemblin. If R-site cleavage is a mechanism for alteration of enzyme specificity, it seems curious that the change acts to restrict (reduced R-site activity) and attenuate (~5- to 10-fold weaker than pPR) activity, rather than potentiate it. However, slowing the cleavage process might help coordinate the maturational changes that ensue, or perhaps reduce the enzyme's ability to attack pPR before its incorporation into nascent capsids. In addition to releasing and mobilizing its catalytic portion within the capsid, and possibly altering its enzymatic activity, R-site cleavage reduces the size of pPR and may be needed by all herpesviruses to help eliminate it from the capsid chamber.

By monitoring processing of the S132A-pPR substrate, we were able to measure and compare the relative kinetics of M-, R-, and I-site cleavage simultaneously, and we determined that pPR and assemblin both attack these sites in the same order observed in cell-based assays (M>R>I) (16, 38). There was some evidence of substrate competition by the cleavage-blocked M-site sequence (native VNA₆₄₃S to mutant VNVS) in the pPR enzyme. Deleting the carboxyl end of the enzyme through that sequence to Gly639 resulted in 10 to 20% better M- and R-site cleavage (Fig. 8C, compare pPR with truncation mutant 639). Truncating both the enzyme and substrate to Gly639 did not dramatically accelerate R-site cleavage (data not shown), indicating that its comparatively lower rate is intrinsic to its primary sequence and/or its context in the substrate, rather than competition from the M site.

Self-interaction of pPR through its scaffolding domain is required for full activity in these protein cleavage assays, as it was in the peptide assays. In both cases, mutation L382A, which disrupts the ability of pPR to form oligomers (4, 19), reduced enzymatic activity but did not eliminate it (Fig. 6 and 7) (4). Mutating just the enzyme reduced its activity about 2-fold. Mutating just the substrate also reduced overall efficiency of the reaction by about 2-fold, revealing a strong contribution of substrate structure to the enzyme-substrate interaction through a distant interface located within the scaffolding domain. With both enzyme and substrate mutated at Leu382, reaction efficiency was reduced about 4-fold (Fig. 7), an observation that might explain the ~4-fold decrease observed in the peptide assay (see Fig. 6B and 9B in reference 4).

The involvement and importance of this scaffolding interaction to the function of pPR are further illustrated by our finding that deletions extending from the carboxyl end of the protein have little effect on enzymatic activity until they ap-

proach the amino-conserved domain. Within 50 to 80 amino acids of that sequence, proteolytic activity weakens and loses its characteristic pPR advantage in R-site cleavage (Fig. 1B and 8D). These results are consistent with our conclusions drawn from peptide assays, namely, that activation of pPR is distinguished from that of assemblin by a requirement for its subunits to interact through their scaffolding sequence and thereby stabilize the interaction at their assemblin dimer interface, which is weaker in the context of pPR than assemblin (4). Even in the context of assemblin, interaction through the dimer interface is relatively weak and is often increased in cleavage reactions by adding Na_2SO_4 , glycerol, or other cosmotropes (10, 11, 20), which may chemically compensate for loss of the pPR scaffolding sequence.

It is expected that the enzymatic and structural differences between pPR and assemblin reported here and before (4, 19) are linked to the biological mechanism of this enzyme. Delivering pPR to the capsid appears to be accomplished and ensured by interactions with other capsid proteins through multiple domains in its scaffolding sequence (e.g., with pAP, through shared amino-conserved and coiled-coil domains; with major capsid protein, through a carboxyl-conserved domain; and probably with the portal protein, through the conserved Pro-Gly-Glu region). However, less is known about the structure, localization, and activation of pPR within the capsid, and important unanswered questions remain. What drives formation of pPR homo-oligomers in the presence of pAP (e.g., preferential self-interaction); are they formed? Is dimerization sufficient to activate pPR? Does pPR form hetero-oligomers with pAP; if so, are they active? Is pPR selectively localized within the capsid (e.g., to vertices)? Answers to these and other related questions will refine existing models and lead to an understanding of how it can be most effectively inhibited. Evidence presented here and before (23), and predictions based on X-ray crystal structure information (1, 6), support there being an active site difference between pPR and assemblin. If substantiated by more direct and rigorous determinations, it will be important to reassess conclusions drawn from screens for inhibitors that were based on assemblin with M site peptide substrates. Truncation mutant $\Delta 469$ -pPR, which lacks half of the scaffolding sequence yet retains the distinguishing structural and enzymatic characteristics of pPR (Fig. 8 and data not presented), may prove valuable in this connection. Because it is less "sticky" and better behaved during chromatography than pPR, $\Delta 469$ -pPR may lend itself more productively to analysis by X-ray crystallography and lead to much-needed atomic-resolution structural information.

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REFERENCES

1. **Batra, R., R. Khayat, and L. Tong.** 2001. Molecular mechanism for dimerization to regulate the catalytic activity of human cytomegalovirus protease. *Nat. Struct. Biol.* **8**:810–817.
2. **Baum, E. Z., et al.** 1993. Expression and analysis of the human cytomegalovirus UL80-encoded protease: identification of autoproteolytic sites. *J. Virol.* **67**:497–506.
3. **Beaudet-Miller, M., et al.** 1996. Viral specific interaction between the human cytomegalovirus major capsid protein and the C-terminus of the precursor assembly protein. *J. Virol.* **70**:8081–8088.
4. **Brignole, E. J., and W. Gibson.** 2007. Enzymatic activities of human cytomegalovirus maturational protease assemblin and its precursor (pPR, pUL80a) are comparable: maximal activity of pPR requires self-interaction through its scaffolding domain. *J. Virol.* **81**:4091–4103.
5. **Brown, J. C., M. A. McVoy, and F. L. Homa.** 2002. Packaging DNA into herpesvirus capsids, p. 111–153. *In* A. Holzenburg and E. Bogner (ed.), *Structure-function relationships of human pathogenic viruses*. Kluwer Academic/Plenum Publishers, New York, NY.
6. **Buisson, M., et al.** 2002. The crystal structure of the Epstein-Barr virus protease shows rearrangement of the processed C terminus. *J. Mol. Biol.* **324**:89–103.
7. **Burck, P. J., et al.** 1994. Human cytomegalovirus maturational proteinase: expression in *Escherichia coli*, purification, and enzymatic characterization by using peptide substrate mimics of natural cleavage sites. *J. Virol.* **68**:2937–2946.
8. **Chan, C. K., E. J. Brignole, and W. Gibson.** 2002. Cytomegalovirus assemblin (pUL80a): cleavage at internal site not essential for virus growth; proteinase absent from virions. *J. Virol.* **76**:8667–8674.
9. **Chen, P., et al.** 1996. Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad. *Cell* **86**:835–843.
10. **Cole, J. L.** 1996. Characterization of human cytomegalovirus protease dimerization by analytical centrifugation. *Biochemistry* **35**:15601–15610.
11. **Darke, P. L., et al.** 1996. Active human cytomegalovirus protease is a dimer. *J. Biol. Chem.* **271**:7445–7449.
12. **Desai, P., and S. Person.** 1996. Molecular interactions between the HSV-1 capsid proteins as measured by the yeast two-hybrid system. *Virology* **220**:516–521.
13. **Gao, M., et al.** 1994. The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. *J. Virol.* **68**:3702–3712.
14. **Gibson, W.** 2008. Structure and formation of the cytomegalovirus virion, p. 187–204. *In* T. Shenk and M. F. Stinski (ed.), *Human cytomegalovirus*. Springer Press, New York, NY.
15. **Holwerda, B. C., et al.** 1994. Activity of two-chain recombinant human cytomegalovirus protease. *J. Biol. Chem.* **269**:25911–25915.
16. **Jones, T. R., et al.** 1994. Proteolytic activity of human cytomegalovirus UL80 proteinase cleavage site mutants. *J. Virol.* **68**:3742–3752.
17. **Kennard, J., F. J. Rixon, I. S. McDougall, J. D. Tatman, and V. G. Preston.** 1995. The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26.5 protein are required for the formation of the capsid shell around the scaffold. *J. Gen. Virol.* **76**:1611–1621.
18. **Liu, F., and B. Roizman.** 1991. The promoter, transcriptional unit, and coding sequence of herpes simplex virus 1 family 35 proteins are contained within and in frame with the UL26 open reading frame. *J. Virol.* **65**:206–212.
19. **Loveland, A. N., N. L. Nguyen, E. J. Brignole, and W. Gibson.** 2007. The amino-conserved domain of human cytomegalovirus UL80a proteins is required for key interactions during early stages of capsid formation and virus production. *J. Virol.* **81**:620–628.
20. **Margosiak, S. A., D. L. Vanderpool, W. Sisson, C. Pinko, and C. C. Kan.** 1996. Dimerization of the human cytomegalovirus protease - kinetic and biochemical characterization of the catalytic homodimer. *Biochemistry* **35**:5300–5307.
21. **Marnett, A. B., A. M. Nomura, N. Shimba, P. R. Ortiz de Montellano, and C. S. Craik.** 2004. Communication between the active sites and dimer interface of a herpesvirus protease revealed by a transition-state inhibitor. *Proc. Natl. Acad. Sci. U. S. A.* **101**:6870–6875.
22. **Matusick-Kumar, L., et al.** 1995. Release of the catalytic domain N(o) from the herpes simplex virus type 1 protease is required for viral growth. *J. Virol.* **69**:7113–7121.
23. **McCartney, S. A., et al.** 2005. Chemical rescue of I-site cleavage in living cells and in vitro discriminates between the cytomegalovirus protease, assemblin, and its precursor, pUL80a. *J. Biol. Chem.* **280**:33206–33212.
24. **Nguyen, N. N., A. N. Loveland, and W. Gibson.** 2008. Nuclear localization sequences in cytomegalovirus capsid assembly proteins (UL80 proteins) are required for virus production: inactivating NLS1, NLS2, or both affects replication to strikingly different extents. *J. Virol.* **82**:5381–5389.
25. **Pelletier, A., F. Do, J. J. Brisebois, L. Lagace, and M. G. Cordingley.** 1997. Self-association of herpes simplex virus type 1 ICP35 is via coiled-coil interactions and promotes stable interaction with the major capsid protein. *J. Virol.* **71**:5197–5208.
26. **Plafker, S. M., and W. Gibson.** 1998. Cytomegalovirus assembly protein precursor and proteinase precursor contain two nuclear localization signals that mediate their own nuclear translocation and that of the major capsid protein. *J. Virol.* **72**:7722–7732.
27. **Preston, V. G., J. A. Coates, and F. J. Rixon.** 1983. Identification and

- characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. *J. Virol.* **45**:1056–1064.
28. Qiu, X., et al. 1996. Unique fold and active site in cytomegalovirus protease. *Nature* **383**:275–279.
 29. Robertson, B. J., et al. 1996. Separate functional domains of the herpes simplex virus type 1 protease: evidence for cleavage inside capsids. *J. Virol.* **70**:4317–4328.
 30. Shieh, H.-S., et al. 1996. Three-dimensional structure of human cytomegalovirus protease. *Nature* **383**:279–282.
 31. Spencer, J. V., W. W. Newcomb, D. R. Thomsen, F. L. Homa, and J. C. Brown. 1998. Assembly of the herpes simplex virus capsid: preformed triplexes bind to the nascent capsid. *J. Virol.* **72**:3944–3951.
 32. Tatman, J. D., V. G. Preston, P. Nicholson, R. M. Elliott, and F. J. Rixon. 1994. Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *J. Gen. Virol.* **75**:1101–1113.
 33. Thomsen, D. R., W. W. Newcomb, J. C. Brown, and F. L. Homa. 1995. Assembly of the herpes simplex virus capsid: requirement for the carboxyl terminal twenty five amino acids of the proteins encoded by the UL26 and UL26.5 genes. *J. Virol.* **69**:3690–3703.
 34. Thomsen, D. R., L. L. Roof, and F. L. Homa. 1994. Assembly of herpes simplex virus (HSV) intermediate capsids in insect cells infected with recombinant baculoviruses expressing HSV capsid proteins. *J. Virol.* **68**:2442–2457.
 35. Tong, L., et al. 1996. A new serine-protease fold revealed by the crystal structure of human cytomegalovirus protease. *Nature* **383**:272–275.
 36. Trang, P., K. Kim, J. Zhu, and F. Liu. 2003. Expression of an RNase P ribozyme against the mRNA encoding human cytomegalovirus protease inhibits viral capsid protein processing and growth. *J. Mol. Biol.* **328**:1123–1135.
 37. Welch, A. R., L. M. McNally, and W. Gibson. 1991. Cytomegalovirus assembly protein nested gene family: four 3'-coterminal transcripts encode four in-frame, overlapping proteins. *J. Virol.* **65**:4091–4100.
 38. Welch, A. R., L. M. McNally, M. R. Hall, and W. Gibson. 1993. Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine. *J. Virol.* **67**:7360–7372.
 39. Welch, A. R., A. S. Woods, L. M. McNally, R. J. Cotter, and W. Gibson. 1991. A herpesvirus maturational protease, assemblin: identification of its gene, putative active site domain, and cleavage site. *Proc. Natl. Acad. Sci. U. S. A.* **88**:10792–10796.
 40. Wittwer, A. J., C. L. Funckes-Shippy, and P. J. Hippenmeyer. 2002. Recombinant full-length human cytomegalovirus protease has lower activity than recombinant processed protease domain in purified enzyme and cell-based assays. *Antiviral Res.* **55**:291–306.
 41. Wood, L. J., M. K. Baxter, S. M. Plafker, and W. Gibson. 1997. Human cytomegalovirus capsid assembly protein precursor (pUL80.5) interacts with itself and with the major capsid protein (pUL86) through two different domains. *J. Virol.* **71**:179–190.